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CONTRACTING ORGANIZATION: Baylor College of Medicine
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13. ABSTRACT (Maximum 200 Words)

Mutations in at least two hedgehog signal transduction network genes leads to defects in mammary gland development. These mutations can cause enhanced hedgehog signaling in some organs. If this mechanism is functioning in the mammary gland, defects should be inhibited or reverted by treatment with specific inhibitors of hedgehog signaling. One such inhibitor is called cyclopamine which causes birth defects in sheep when ingested during pregnancy. We are using slow release pellets containing cyclopamine to test whether Smo, Ptc1 or Gli2-induced developmental defects are inhibited or reverted by treatment. As controls we are also testing the in vivo effect of cyclopamine treatment of preneoplastic growths presumably caused by genetic alterations independent of hedgehog signaling. The effect of cyclopamine on normal mammary development and function is also being examined. Finally, we are testing the effect of cyclopamine on a series of human mammary epithelial cell lines for changes in their growth behavior. If cyclopamine can slow or prevent neoplastic growth, this class of inhibitors may be useful in breast cancer treatment or prevention.

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Introduction

The hedgehog signal transduction network mediates cell-cell communication during normal embryonic development. Genetic mutation of hedgehog network genes can cause severe birth defects, basal cell carcinoma of the skin, and other tumors including medulloblastomas and glioblastomas of the brain.

Our recent work demonstrates a role for hedgehog signaling in mammary cancer and normal mammary gland development in the mouse. Loss-of-function mutations in two hedgehog network genes, *Patched-1 (Ptc-1)* and *Gli-2*, cause cancer-like lesions that closely resemble human ductal carcinoma *in situ* (DCIS. The lesions become invasive with age but, like basal cell carcinoma and medulloblastoma, are not stable upon transplantation.

The specific mechanism by which mutations in the hedgehog network lead to mammary lesions is not known. In basal cell carcinoma, loss of *Ptc-1* function or overproduction of either *Smoothened (Smo)* or one of the three hedgehog proteins, *Sonic hedgehog (Shh)*, leads to tumors. If the mechanics of the hedgehog signaling network are conserved between skin and mammary gland, a specialized skin derivative, these observations lead to the following hypothesis:

> *mutation of either the Ptc-1 or Gli2 genes results in improper activation of the signaling network via inappropriate activity of either Smo or the hedgehog proteins themselves. This improper signaling leads to loss of normal growth control and mammary lesion formation.*

We will use two approaches to test this hypothesis. First, we will construct a transgenic mouse line that expresses a constitutively activated form of *Smo* that signals independently of hedgehog protein binding and cannot be inhibited by *Ptc-1*. When expressed in the skin, this form of *Smo* promotes skin tumors. We expect that altered *Smo* gene will promote tumor formation and will therefore identify *Smo* as a mammary oncogene.

Second, we will use specific inhibitors of hedgehog protein signaling and determine whether these agents can reverse *Ptc-1-* , *Gli2* - or *Smo*-induced mammary lesions or lessen their severity. As controls we will examine the effects of these agents on well-characterized mouse tumor models. Agents will be delivered via surgical implantation of slow-release plastic pellets within the gland. Mammary glands will be examined for changes in structure and effects on lesion growth. Glands will also be assayed for changes in cell division (DNA synthesis) and cell death (apoptosis) as well as in expression of hedgehog network genes in response to treatment.

In addition, we will test the effect of these specific inhibitors on a panel of human breast cancer cell lines. It is possible that these inhibitors may affect the growth characteristics of a subset of human cancers thereby implicating the hedgehog network as a contributory factor in breast cancer onset or progression.

If hedgehog activity is responsible for, or participates in lesion formation or progression, we anticipate that treatment will reverse the formation of lesions or slow their growth. Such findings would justify expanded pre-clinical and clinical investigation of related hedgehog signaling inhibitors for potential therapeutic value in the treatment or prevention of human breast cancers.

Summary of results

Task 1. To determine whether constituitive activation of hedgehog signaling leads to mammary lesions in transgenic mice using an activated form of *Smo* that signals independently of hedgehog protein binding and is unresponsive to *Ptc-1* inhibition.

a. Generation of *MMTV:Smo* transgenic mouse line

Progress: Our collaboration with Dr. DeMayo, Director of the Baylor Mouse Genetics Core Facility has worked out wonderfully. After several unsuccessful rounds of injections, we changed strains to the FVB inbred background and now have six MMTV-SmoM2 founder lines breeding. Four of these lines express the transgene at both the RNA and protein levels. All four lines of mice show consistent alteration of end bud morphology and histoarchitecture, as well as ductal dysplasias similar to those observed previously in the Ptc1/+ mice.

We have been able to develop an immunofluorescence protocol to detect mouse and human Smo protein in tissue.

Work with TOSK Inc. (Santa Cruz, CA) to generate transgenic mice has been suspended.

b. Breeding and tissue harvest for phenotypic and gene expression analysis

Our initial focus has been ductal development. Tissue has been collected from four transgenic lines thus far – all showing similar defects in ductal development. We have also set aside mice for long-term tumor formation studies.

c. Phenotypic and gene expression analysis.

Assays to detect Ptc1, Ptc2 and Smo proteins have been developed. Microarray work is pending collection of an adequate number of samples at the desired developmental time points. These data will be compared with similar data generated for the Ptc1/+ and Ptc1 overexpressing transgenic models we maintain currently.

Task 2. To test the *in vivo* effect of specific hedgehog protein inhibitors on hedgehog network-induced lesions and the normal mammary gland.

d. Implantation and analysis of inhibitors on *Smo* -induced mammary lesions

Pending selection of appropriate of MMTV:Smo transgenic mouse lines and phenotypic analysis

Task 3. To test the *in vivo* effect of specific hedgehog protein inhibitors on hedgehog-independent lesions

We are now entering into an expanded collaboration with Curis, Inc. which recently developed small molecule inhibitors of Smo, on in vivo studies in transgenic mouse models (MMTV-c-ErbB2 and MMTV-Wnt1 models). Curis has agreed to provide adequate drug to complete these studies. However, a recent partnership with Genentech requires that we amend our current MTA with Genentech (in collaboration with Dr. Fred de Sauvage who is listed in this grant). This is in progress

Experiments with the HAN lines have been suspended indefinitely. The HAN tissue lines cannot be brought into the mouse facility in which our animals are housed and we have identified transgenic models that will serve our purposes better.

Task 4. To test the effect of hedgehog inhibitors on the growth and morphology of human breast cancer cell lines *in vitro.*

a. Acquire and establish human mammary epithelial cell lines.

Cell lines established and under study are:

1. MCF10A	"NORMAL"
2. MCF12A	"NORMAL"
3 MCF7	CANCER
4. T47D	CANCER
5. MDA231	CANCER
6. MDA468	CANCER
7. Human Mammary Epithelial Cells	"NORMAL"

b. Test effect of inhibitors on growth and morphology of human cell lines

We have completed dose-response growth curves for MCF7 (ER positive), MDA231, and MCF10A which show differential sensitivity to cyclopamine, KAAD-cyclopamine, and the Curis compounds.

We have completed colony forming assays for MCF7. Colony forming potential is decreased in a dose-dependent manner.

We have completed proliferation (MTT and BrdU incorporation) and apoptosis assays (TUNEL) for MCF7. Inhibitors affect proliferation rates but do not affect apoptosis rates.

Results are being prepared for publication. Based on these data, we are moving into in vivo preclinical models of breast cancer. A phase I clinical trial in patients with advanced breast cancer is in the design phase in collaboration with Dr. Jenny Chang of the Baylor Breast Center.

Key Research Accomplishments

With the exception of Task 3, the in vivo efficacy of compounds on mammary hyperplasias and tumors, the major goals of this project have been met. Results are entirely consistent with the initial hypothesis and strongly indicate that hedgehog signaling inhibitors should be considered for development in the treatment or prevention of breast cancer.

Cyclopamine and related compounds inhibit breast cancer cell proliferation and colony forming potential in a dose-dependent manner. Non-teratogenic steroidal alkaloids do not affect cell growth except at the high end of the dosages tested (50μM). Compounds affect proliferation but do not affect apoptosis.

Generation and characterization of MMTV-Smo transgenic lines. Phenotypic analysis is consistent with the stated hypothesis that altered hedgehog signaling leads to ductal dysplasia.

Reportable Outcomes

Publications and Manuscripts

1. The *Hoxd10* Homeobox Gene Regulates Lactogenesis in the Mouse Mammary Gland. Michael T. Lewis[1*], Yael Friedman[2], Phyllis Strickland[3], Ellen M. Carpenter[4], and Charles W. Daniel[3] (submitted)
2. McManaman, J.L., Palmer, C.A., Zabaronik, W., Rizzoli, S., Fischer, A., Hanson, L., Lewis, M.T. and Neville, M.C. Regulation of lipid storage and secretion in the mouse mammary gland during secretory differentiation. (submitted)
3. Michael T. Lewis[1,2*], James McManaman[2], Linda Hanson[2], Valerie Sawicki[2], Gary B. Silberstein[3], Susan G. Hilsenbeck[1], and Margaret C. Neville[2] Hedgehog Signaling is Required for Functional Differentiation of the Mouse Mammary Gland (in preparation)

Presentations

1. Lewis, M.T. Baylor College of Medicine (Various)
2. Lewis, M.T. MD Anderson (Nov 2002)
3. Lewis, M.T. Think Tank 13 (March 2003)

Employment received and research opportunities.

ACTIVE

DAMD17-03-1-0571 (Lewis) 7/15/03-7/14/04
DOD $75,000
Combining Cell and Gene Therapy For Treatment Of Early Stage Breast Cancer

17580 (Lewis) 7/15/03-7/14/0
Susan Love MD Breast Cancer Research Foundation $19,500
Development of an Intraductal Cell and Gene Therapy Approach for Treatment of Early Stage Breast Cancer

PENDING

P01 CA30195 (Osborne/Lewis) 12/1/03-11/30/08
NIH $187,352
Novel Gene Networks in Breast Development and Cancer, Project 4: The Ptc1 Hedgehog Receptor in Mammary Ductal Development and Progression to Neoplasia

R01 CA30195 (Lewis) 6/1/04-7/31/09
NIH $250,000
The Ptc1 Hedgehog Receptor in Mammary Ductal Development and Progression to Neoplasia

BC030850 (Lewis) 6/1/04-5/31/07
DOD $100,000
The role of the Gli2 transcription factor in neoplastic progression

No number 6/1/04-5/31/07
Susan G. Komen Foundation $75,000
In vivo analysis of the *Gli2* transcription factor gene, a candidate tumor suppressor in mammary stroma.

Conclusions

- Ductal development is not dependent on hedgehog signaling, and signaling must be inhibited actively for normal development to occur.
- Cyclopamine and related compounds are effective in inhibiting breast cancer cell growth in vitro. Compounds affect proliferation but not apoptosis.
- Ectopic hedgehog signaling leads to ductal dysplasia

Appendices

1. Curriculum vitae for Michael T. Lewis

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Michael T. Lewis	**Assistant Professor**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
College of William and Mary, Williamsburg, Virginia USA	B.S.	1982-1986	Biology
University of California, Santa Cruz, California USA	Ph.D.	1989-1995	Biology
University of California, Santa Cruz, California USA	Post-doc.	1995-1998	Biology
University of Colorado, Denver, Colorado USA	Post-doc.	1999	Physiology and Biophysics

A. Positions and Honors

7/01-present **Assistant Professor** – Baylor College of Medicine Breast Center and the Department of Molecular and Cellular Biology, Houston, TX 77030.
Molecular genetics of mammary gland development and breast cancer in mouse and human. Focus on the function of the *hedgehog* signal transduction network and homeobox genes.

6/99-6/01 **Instructor** – University of Colorado School of Medicine, Denver, CO 80262. Department of Physiology and Biophysics.
Molecular genetics of mammary gland development and breast cancer in mouse and human. Focus on the function of the *hedgehog* signal transduction network and homeobox genes.

1/99-6/99 **Postdoctoral Research Associate** – University of Colorado School of Medicine, Denver, CO 80262. Department of Physiology and Biophysics. Laboratory of Dr. Peggy Neville.
Molecular genetics of mammary gland development and breast cancer in mouse and human. Focus on the function of the *hedgehog* signal transduction network and homeobox genes.

7/95-12/98 **Post Graduate Researcher** – University of California, Santa Cruz, CA 95064. Laboratory of Dr. Charles Daniel.
Molecular genetics of mammary gland development and breast cancer in mouse and human. Focus on the function of the *hedgehog* signal transduction network and homeobox genes.

9/89-6/95 **Teaching Assistant** – University of California. Department of Biology. Santa Cruz, CA 95064.
In: Genetics (5 quarters), Howard Hughes Summer Institute for Molecular Biology (5 quarters), Genetics Laboratory, Cell Biology, Neurobiology, and Virology.

9/89-6/95 **Graduate Researcher** – University of California. Department of Biology. Santa Cruz, CA 95064. Laboratory of Dr. Jerry Feldman.
Molecular genetics and evolution of circadian (daily) rhythms in the filamentous fungus *Neurospora crassa*

7/88-8/89 **Research Scientist** – National Biomedical Research Foundation – Protein Information Resource (NBRF-PIR).

10/86-7/88 **Biologist** – National Biomedical Research Foundation – Protein Information Resource (NBRF-PIR).3900 Reservoir Rd., N.W., Washington, D.C., 20007.

The NBRF-PIR Protein Sequence Database is an internationally used bioinformatics resource. Research directed toward evolutionary and functional characterization of proteins and development of advanced sequence analysis protocols.

Sigma Xi Charter member - Santa Cruz chapter; San Antonio Breast Cancer Symposium Organization Committee Member (2001-2002); University of California Breast Cancer Research Program - Pathogenesis Study Section Member (2001-2002)

Invited Talks: Genentech Inc. (7/98); Lawrence Berkeley National Laboratory (8/98); University of Miami School of Medicine (8/98); Gordon Research Conference on Mammary Gland Biology (6/99); National Cancer Institute (11/99); University of Colorado Health Sciences Center (3/00); Baylor Breast Center (8/00); Gordon Research Conference on Mammary Gland Biology (6/01); Think Tank 12 (3/02); MD Anderson Cancer Center (11/02); Think Tank 13 (1/03)

B. Selected Peer-reviewed publications

RESEARCH ARTICLES:

Lewis, M.T., Ross, S., Strickland, P.A., Sugnet, C., Jimenez, E., Hui, C-c. and Daniel, C.W. (2001) The *Gli2* transcription factor is required for normal mouse mammary gland development. Dev. Biol. 238:133-144

Lewis, M.T., Ross, S., Strickland, P.A., Sugnet, C., Jimenez, E. Scott, M.P. and Daniel, C.W. (1999) Defects in mouse mammary gland development caused by conditional haploinsufficiency of *Patched-1 (Ptc1)*. Development 126:5181-5193

Lewis, M.T., Ross, S., Strickland, P.A., Snyder, C.J. and Daniel, C.W. (1999) Regulated expression patterns of *IRX-2*, an Iroquois-class homeobox gene, in the human breast. Cell Tissue Res. 296:549-554

Lewis, M.T. and Feldman, J.F. (1998) Genetic mapping of the *band (bd)* locus of *Neurospora crassa*. Fungal Genet. Newsl. 45: 21.

Lewis, M.T., Morgan, L.W., and Feldman, J.F. (1997) Analysis of *frequency (frq)* clock gene homologs: evidence for a helix-turn-helix transcription factor. Mol. Gen. Genet. 253:401-414

Lewis, M.T. and Feldman, J.F. (1996) Evolution of the *frequency (frq)* clock locus in fungi. Mol. Biol. Evol. 13:1233-1241

Lewis, M.T., and Feldman, J.F. (1993) The putative *frequency (frq)* clock protein of *Neurospora crassa* contains sequence elements that suggest a nuclear transcriptional regulatory role. Protein Seq. Data Anal. 5: 315-323

Ron, D., Zannini, M., **Lewis, M.T.**, Wickner, R.B., Hunt, L.T., Graziani, G., Tronick, S.R., Aaronson, S.A., and Eva, A. (1991) A region of proto-*dbl* essential for its transforming activity shows sequence similarity to a yeast cell cycle gene, *CDC24*, and the human breakpoint cluster gene, *bcr*. The New Biologist 3: 372-379

Lewis, M.T., Hunt, L.T., and Barker, W.C. (1989) Striking sequence similarity among sialic acid-binding lectin, pancreatic ribonucleases, and angiogenin: possible structural and functional relationships. Protein Seq. Data Anal. 2: 101-105

REVIEW ARTICLES:

Lewis, M.T. (2001) Hedgehog signaling in mammary gland development. J. Mammary Gland Biol. Neoplasia 6:53-66

Nguyen, D., Beeman, N., **Lewis, M.T.**, Schaack, J. and Neville, M.C. (2000) Intraductal injection into the mouse mammary gland. Methods in Mammary Gland Biology and Breast Cancer Research. M.M. Ip and B.B. Asch (eds.) Kluwer Academic/Plenum Publishers, New York. 259-270

Lewis, M.T. (2000) Homeobox genes in mammary gland development and neoplasia. Breast Cancer Research 2: 158-169

DATA COLLECTIONS:

Protein Sequence Database. Barker, W.C., Hunt, L.T., George, D.G., Yeh, L.S. Chen, H.R., Blomquist M.C., Seibel-Ross, E.I., Elzanowski, A., Bair, J.K., **Lewis, M.T.**, Marzec, C.R., Davalos, D.P. and Ledley, R.S. National Biomedical Research Foundation, Washington, D.C. (Updated quarterly) (1986-1989).

C. Research Support.

Title: Hedgehog Signal Transduction Inhibitors in Breast Cancer Treatment and Prevention
Agency: Department of Defense (IDEA) 17 00 1 0477
Role: PI
Period: 7/1/00-6/30/03
The major goals of this project are to determine whether constitutive activation of hedgehog signaling can lead to mammary lesions in transgenic mice using an activated form of *Smo* that signals independently of hedgehogs and is unresponsive to *Ptc-1* inhibition, to test the *invivo* effect of specific hedgehog protein inhibitors on hedgehog network-induced lesions and the normal mammary gland, to test the *invivo* effect of specific hedgehog protein inhibitors on hedgehog-independent lesions, and to test the effect of hedgehog inhibitors on the growth and morphology of human breast cancer cell lines *in vitro.*

Title: SPORE in Breast Cancer-Project 5: Genetic Expression Profile of Taxotere Versus AC Sensitivity
Agency: NIH (P50 CA58183)
Role: Co-Investigator
Period: 12/1/02-11/30/07
The main goal of this project is to identify, confirm and validate prospectively and retrospectively, two genetic pathways involved in the sensitivity and resistance of the two main treatment regimens in breast cancer, Taxotere (T) and Adriamycin plus cyclophosphamide (AC).

Title: Development of an Intraductal Cell and Gene Therapy Approach for Treatment of Early Stage Breast Cancer
Agency: Susan Love MD Breast Cancer Research Foundation
Role: PI
Period: Pending Funding
The goal of this pilot project is to perform a "proof of principle" experiment to determine whether a patient's own breast cells can be removed, genetically modified to perform a therapeutic function, and reintroduced intraductally to survive long-term to combat cancer.

Title: Induction of mammary cancer by signaling molecules
Agency: NCI (R01 CA85736 Anderson, PI)
Role: Co-Investigator*
Period: 4/1/00-6/30/01 (3/31/05)
The major goals of this project are to determine whether constitutive activation of either the prolactin receptor or one of its downstream effectors (Akt) will contribute to neoplastic progression or developmental defects in the mouse mammary gland.

*Although he has changed institutions, Dr. Lewis has continued to collaborate on this study; however, not as a co-investigator.

Title: Functional Development of the Mammary Gland

Agency: NIH (PO1 HD38129 Neville, PI)
Role: Co-Project Leader/Animal Core Director
Period: 7/1/00-6/30/01 (6/30/05)

*Before relocating to Baylor College of Medicine, Dr. Lewis devoted 20% time as Co-Principal Investigator with Dr. Dean Edwards (UCHSC Department of Pathology) on a project to define the mechanisms of inhibition of milk secretion by progesterone during pregnancy and 20% time as the Animal Core Director for the program project group. He continues to collaborate with the group from the University of Colorado.